HILIC Chromatography: When and How?

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Applications Engineer
LC Columns and Consumables Technical Support
January 25, 2022
Agenda

1. What is HILIC and when should you consider it?
2. HILIC method development
   - Agilent HILIC column options
   - Mobile phase considerations
3. Tips and tricks for successful HILIC column use and care
   - Column equilibration
   - Sample solvent compatibility
   - Inert HILIC solution for metal-sensitive compounds
4. Summary
A water layer is adsorbed onto the polar silica surface, creating a liquid/liquid extraction system.

1. Polar analytes can partition into and out of the water layer, with more polar analytes having a stronger interaction.
2. Charged polar analytes can also undergo ion exchange with the silica surface.

Elution is typically from least to most polar, which is the opposite of RPLC.

Solvent strengths in HILIC mode are: THF < acetone < acetonitrile < isopropanol < ethanol < methanol < water.
What Is HILIC and When Should You Consider It?

HILIC compared to RPLC
### What Is HILIC and When Should You Consider It?

**HILIC complements RPLC**

<table>
<thead>
<tr>
<th>Reversed-Phase LC</th>
<th>Hydrophilic Interaction LC (HILIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonpolar stationary phase (for example, C18)</td>
<td>Polar stationary phase (for example, silica)</td>
</tr>
<tr>
<td>Polar mobile phase H₂O/CH₃OH, H₂O/CH₃CN</td>
<td>Polar mobile phase H₂O/CH₃CN</td>
</tr>
<tr>
<td>Decrease retention by decreasing polarity of mobile phase</td>
<td>Decrease retention by increasing polarity of mobile phase</td>
</tr>
<tr>
<td>H₂O ↓ = retention ↑</td>
<td>H₂O ↑ = retention ↓</td>
</tr>
<tr>
<td>CH₃CN ↑ = retention ↓</td>
<td>CH₃CN ↓ = retention ↑</td>
</tr>
<tr>
<td>Polar to nonpolar</td>
<td>Nonpolar to polar</td>
</tr>
</tbody>
</table>
Find the Best Column to Retain and Separate All Analytes

HILIC retains amino acids and separates isobars, while RPLC can’t

HILIC: Poroshell 120 HILIC-Z

RPLC: Poroshell 120 PFP

Separation of isobars leucine/isoleucine
Both RPLC and HILIC are able to retain and separate water-soluble vitamins.
What Is HILIC and When Should You Consider It?

When to choose which separation mode for your sample

01. Find the best column to retain and separate all analytes.

02. Consider the sample: analyte solubility and sample solvent

03. Ensure reliable detection of your sample
What Is HILIC and When Should You Consider It?

When to choose which separation mode for your sample

01
Find the best column to retain and separate all analytes.

02
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03
Ensure reliable detection of your sample
What Is HILIC and When Should You Consider It?

InfinityLab Poroshell 120 offers a broad portfolio to suit your needs

<table>
<thead>
<tr>
<th>Best all around</th>
<th>Best for low pH mobile phases</th>
<th>Best for high pH mobile phases</th>
<th>Best for alternative selectivity</th>
<th>Best for more polar analytes</th>
<th>Chiral</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC-C18</td>
<td>1.9 µm, 2.7 µm, 4 µm</td>
<td>S</td>
<td>Bonus-RP 2.7 µm</td>
<td>SB-Aq 1.9 µm, 2.7 µm, 4 µm</td>
<td>Chiral-V 2.7 µm</td>
</tr>
<tr>
<td>EC-C8</td>
<td>1.9 µm, 2.7 µm, 4 µm</td>
<td>S</td>
<td>PFP 1.9 µm, 2.7 µm, 4 µm</td>
<td>EC-CN 2.7 µm</td>
<td>Chiral-T 2.7 µm</td>
</tr>
<tr>
<td>Phenyl-Hexyl</td>
<td>1.9 µm, 2.7 µm, 4 µm</td>
<td>S</td>
<td>HILIC 1.9 µm, 2.7 µm, 4 µm, pH range 0.0-8.0</td>
<td></td>
<td>Chiral-CD 2.7 µm</td>
</tr>
<tr>
<td>HILIC</td>
<td>2.7 µm, pH range 1.0-7.0</td>
<td></td>
<td>HILIC-Z 1.9 µm, 2.7 µm, 4 µm, pH range 2.0-12.0</td>
<td></td>
<td>Chiral-CF 2.7 µm</td>
</tr>
</tbody>
</table>

RP chemistries for polar analytes

HILIC chemistries

Chiral
What Is HILIC and When Should You Consider It?

When to choose which separation mode for your sample

01 Find the best column to retain and separate all analytes.

02 Consider the sample: analyte solubility and sample solvent.

03 Ensure reliable detection of your sample.
Analyte Solubility and Sample Solvent

Strong injection solvents distort peak shapes for HILIC and RPLC

**HILIC: Glufosinate**
- 4 µL injection of Glufosinate, 100 ppb, InfinityLab Poroshell HILIC-Z, 2.1x100 mm; Temperature: 30 ºC; Flowrate: 0.6 mL/min, Mobile phase A: 10 mM ammonium acetate, pH 9, Mobile phase B: 100 mM ammonium acetate, pH 9 in 90% ACN (final concentration: 10 mM), Gradient: 90% B => 60% B in 10 minutes, System: Agilent 6490 LC/QQQ

**in 100% H₂O**
- Peak splitting

**in 1:1 CH₃CN/H₂O**
- Peak splitting

**RPLC: Pyridoxine (vitamin B6)**
- 0.5 µL injection of 13 µg/mL pyridoxine, A: H₂O; B: CH₃CN; D: 200 mM ammonium acetate + 0.2% acetic acid, pH ~5.3; 0.5 mL/min; Gradient: 0% B for 1 min, 0-25% B in 8 min, hold 5% D constant throughout analysis; Column: 25 ºC, 2.1 x 100 mm, 2.7 µm Agilent InfinityLab Poroshell 120 Phenyl-Hexyl; Detection: Ultivo TQ/MS ESI+ dMRM

**in 100% H₂O**

**in 9:1 CH₃CN/H₂O**
- Peak splitting

**in 1:1 CH₃CN/H₂O**

**Peak splitting**

January 25, 2022
Analyte Solubility and Sample Solvent

Strong solvent effects are greater with larger injection volumes

Sample solvent: **100% water**

1 µL

2 µL

3 µL

4 µL

5 µL

Sample solvent: **50:50 CH₃CN/H₂O**

4 µL

5 µL

Sample solvent: **80:20 CH₃CN/H₂O**

10 µL

Sample: Glufosinate, 100 ppb
Column: InfinityLab Poroshell HILIC-Z, 2.1 x 100 mm
Temperature: 30 ºC
Flowrate: 0.6 mL/min
Mobile phase A: 10 mM ammonium acetate, pH 9
Mobile phase B: 100 mM ammonium acetate, pH 9 in 90% ACN (final concentration: 10 mM)
Gradient: 90% B => 60% B in 10 minutes
System: Agilent 6490 LC/QQQ

Note: System required phosphoric acid wash
Ensure samples are completely soluble

HILIC

Nicotinic Acid
0.4 µg/mL

in H₂O
Peak Area = 562

in 9:1 CH₃CN/H₂O
Peak Area = 570

Thiamine
0.4 µg/mL

in H₂O
peak area = 8112

in 9:1 CH₃CN/H₂O
peak area = 4575

Cyanocobalamin
0.4 µg/mL

in H₂O
peak area = 104

in 9:1 CH₃CN/H₂O
peak area = 56

~50% sample lost due to poor solubility in CH₃CN
What Is HILIC and When Should You Consider It?

When to chose which separation mode for your sample

01
Find the best column to retain and separate all analytes.

02
Consider the sample: analyte solubility and sample solvent

03
Ensure reliable detection of your sample
Ensure Reliable Detection of Your Sample

Choose a Detector that Can Analyze Compounds of Interest

UV, VIS absorbance
- For light-absorbing compounds

Refractive index
- Universal detection, but poor sensitivity; can only run isocratic

Evaporative light scattering
- For nonvolatile analytes

Mass spectrometer
- Low limits of detection based on molecular weight

Fluorescence
- For compounds that fluoresce or can be derivatized to do so

Can not detect Vitamins B7 and B5 by UV (260 nm)
HILIC pairs well with LC/MS and can improve sensitivity compared to RPLC for opioid metabolites.

Columns used were 2.1 x 100 mm, 1.8 μm; A: 10 mM ammonium formate pH 3.2 in water, B: acetonitrile/100 mM ammonium formate pH 3.2 in water (9:1), isocratic elution, 0.4 mL/min, 2 μL injection of 1 μg/mL each of morphine-3-β-D-glucuronide, and morphine-6-β-D-glucuronide; 25 °C, MS Source: ESI+, 200 V, 250 °C, 11 L/min., 30 psi, 4000 V; SIM: 462, Frag 170 V, Agilent publication: 5991-0245

Ensure Reliable Detection of Your Sample

Agilent ZORBAX RRHD Eclipse Plus C18, 10% CH₃CN, isocratic
S/N₆G=37

Agilent ZORBAX RRHD HILIC Plus 70% CH₃CN, isocratic
S/N₆G=144
Reversed-Phase LC and UV Detection are Compatible with a Wider Range of Mobile Phases, Especially at Low pH

<table>
<thead>
<tr>
<th>Mobile Phase</th>
<th>Useable pH/Range</th>
<th>Recommended for HILIC?</th>
<th>Recommended for MS?</th>
<th>Recommended for RPLC and UV?</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFA</td>
<td>&lt;1.5</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1.1-3.1</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>&lt;2.8</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Acetic Acid</td>
<td>&lt;3.8</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Formate</td>
<td>2-8-4.8</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Acetate</td>
<td>3.8-5.8</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Carbonate</td>
<td>5.4-7.4</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Phosphate</td>
<td>6.2-8.2</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Bicarbonate</td>
<td>6.6-8.6</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Ammonia</td>
<td>8.2-10.2</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Phosphate</td>
<td>11.3-13.3</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>
What Is HILIC and When Should You Consider It?

Summary of when to use which separation mode

01
Find the best column to retain and separate all analytes.

RPLC cannot retain all polar/ionized analytes. HILIC may work for these.

Some analytes can be retained and separated equally well in both modes of LC.

02
Consider the sample: analyte solubility and sample solvent

Injecting strong solvent in both RPLC and HILIC will negatively affect chromatographic quality.

Strong solvent effects get worse with larger injection volumes.

Polar compounds are generally more soluble in water than acetonitrile, which is good for RPLC.

It's a balancing act

03
Ensure reliable detection of your sample

Ensure analytes are compatible with detector choice.

HILIC can improve LCMS analyses due to more volatile mobile phases.

UV and RPLC are compatible with a wider variety of mobile phases, which may improve analyte retention and separation.
## Other Techniques for Polar Compounds

<table>
<thead>
<tr>
<th>Technique</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion Pairing</td>
<td>Fast. Uses standard system and reverse phase columns.</td>
<td>Often contaminates system, reagents can cause ion suppression, restricted to only positive or only negative mode MS.</td>
</tr>
<tr>
<td>Ion Chromatography</td>
<td>Well understood mechanism, established for over 40 years.</td>
<td>Slower than modern HPLC, expensive systems and consumables, cannot resolve cations and anions simultaneously, difficult to make MS-compatible.</td>
</tr>
<tr>
<td>Ion Exchange</td>
<td>Strong retention and separation</td>
<td>Slower than HPLC, cannot resolve cations and anions simultaneously, difficult to make MS-compatible.</td>
</tr>
<tr>
<td>Derivatization</td>
<td>Tailored selectivity, adds chromophore or fluorophore</td>
<td>Lengthy sample preparation, repeatability issues.</td>
</tr>
</tbody>
</table>
What Is HILIC and When Should You Consider It?

Advantages

- Uses a standard system and solvent, just swap columns
  - Easily adopted by labs currently performing reverse phase analysis

- Retains cations, anions, and polar neutrals
  - Widely applicable across all major polar samples

- Fully MS compatible
  - Operate in positive or negative mode with high sensitivity
HILIC Method Development
And common application areas
HILIC Method Development

InfinityLab Poroshell 120 HILIC column options

**HILIC**
- Bare silica chemistry
- For very simple mixtures, low column bleed

**HILIC-Z**
- Proprietary zwitterionic chemistry, high pH stable
- **The most modern and robust column – start method development here**
- PEEK-lined version available

**HILIC-OH5**
- Brushed fructan chemistry
- Alternative selectivity

Best for polar analytes

InfinityLab Poroshell
HILIC
1.9 µm, 2.7 µm, 4 µm

InfinityLab Poroshell
HILIC-Z
1.9 µm, 2.7 µm, 4 µm

InfinityLab Poroshell
HILIC-OH5
2.7 µm
HILIC Method Development

Mobile phase considerations

<table>
<thead>
<tr>
<th>Recommendations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Organic solvent concentration</strong></td>
</tr>
<tr>
<td>• Solvent strength in HILIC mode: ( THF &lt; \text{Acetone} &lt; CH_3CN &lt; IPA &lt; EtOH &lt; MeOH &lt; H_2O )</td>
</tr>
<tr>
<td>• ( H_2O ) must be present — need &gt;3% ( H_2O ) for hydration of silica</td>
</tr>
<tr>
<td>• Mobile phase will typically be &gt;50% acetonitrile</td>
</tr>
<tr>
<td><strong>Ionic strength of buffer</strong></td>
</tr>
<tr>
<td>• Concentration of (salt) buffer increases strength</td>
</tr>
<tr>
<td>• Different anions and cations may also affect analyte retention</td>
</tr>
<tr>
<td><strong>Type of buffer</strong></td>
</tr>
<tr>
<td>• Acetates and formates are good, soluble in CH3CN—also MS friendly</td>
</tr>
<tr>
<td>• Phosphate salts are bad due to low CH(_3)CN solubility</td>
</tr>
</tbody>
</table>

More information

For more HILIC method development tips, see this publication: 5991-9271EN
HILIC Method Development

Less CH₃CN makes a HILIC mobile phase stronger, causing less retention

Column used was 2.1 x 150 mm, 2.7 μm Agilent InfinityLab Poroshell 120 HILIC-Z (PEEK lined); A: 100 mM pH 3 ammonium formate in Water, B: Acetonitrile, x % B, isocratic elution, 0.25 mL/min, 30 °C, 1 μL injection of toluene, cytosine, uracil QC mixture, 254 nm
HILIC Method Development

Starting mobile phases

**Mobile phase A (strong phase, H₂O):**

- Typical buffer concentration: 5 to 30 mM
  - 10 to 20 mM is most common
- Ammonium formate, pH 3
- Ammonium acetate, pH 4-5

**Mobile phase B (weak phase, CH₃CN):**

- Buffer concentration should match mobile phase A for improved reproducibility
- Adding 10% water in ACN is generally recommended for improved solubility and faster re-equilibration
- Pure MeOH is too strong a solvent for most HILIC separations. Mixed with ACN in small quantities (<15%), it can be used to change selectivity slightly

### Basic analytes

- Ammonium formate, pH 3
- Ammonium acetate, pH 4-5
- Ammonium acetate, pH ~7
  - Ammonium acetate solution is near pH 7, before adjusting with other modifiers
  - Not a true buffer, but still commonly used at mid-pH

### Acidic analytes

- Ammonium acetate or formate, pH 9-10
  - Can be formate or acetate because the ammonium ion is buffering
  - HILIC-Z only
- Ammonium hydroxide, pH 10-11
  - HILIC-Z only

### Sugars

- Phosphate buffers are not recommended *

*Note: Phosphates have low solubility in high % ACN (1-30 mM). Always test solubility before running. Never run in >80% ACN to avoid precipitation.
In HILIC mode, ionizable compounds are better retained when they are ionized

- Acids at high pH
- Bases at low pH

Once the analyte is fully ionized, retention should stabilize

- Note: If other retention mechanisms are occurring, this may not be true

**Biotin pKa = 4.5**

**Nicotinic acid pKa = 4.8**

**Pantothenic acid pKa = 4.3**

Mobile phase A: H₂O, B: CH₃CN, D: varies, 200 mM ammonium formate or acetate; Flow rate: 0.5 mL/min; Gradient: 95% B for 1 min, 95-85% B in 9 min, hold 5% D constant throughout analysis, 5 min post run; Injection: 0.5 µL of 13.3 µg/mL each in CH₃CN/H₂O 19:1; Column: 25°C, 2.1 x 100 mm, 2.7 µm Agilent InfinityLab Poroshell 120 HILIC-Z; Detection: Ultivo TQ/MS ESI+ dMRM
## Common starting conditions for HILIC method development

<table>
<thead>
<tr>
<th>Method Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Column</strong></td>
<td>Agilent InfinityLab Poroshell 120 HILIC-Z</td>
</tr>
</tbody>
</table>
| **Buffer**       | • **Acidic analytes**: mid to high pH (HILIC-Z only)  
                        • **Basic analytes**: low to mid pH  
                        • **Mixed analytes**: mid pH |
| **Isocratic**    | Column equilibration is faster as you move from high to low aqueous  
                        • 50% ACN – Column wash (typically no retention)  
                        • 70% ACN – Very polar analytes  
                        • 80% ACN – Polar analytes, mixtures  
                        • 90% ACN – Less polar analytes separation |
| **Gradient**     | • 90% → 50% ACN – Scouting gradient  
                        • Isocratic holds or shallow gradients (1-3% per min) recommended for critical pair separation |
Tips and Tricks

For successful HILIC column use and care
## Tips and Tricks for Your HILIC separations

### Considerations on solvent and sample handling

<table>
<thead>
<tr>
<th>Requirement</th>
<th>Impact</th>
</tr>
</thead>
</table>
| Add 10% aqueous to your organic solvent | • Buffer solubility increases drastically with addition of 10-20% water  
• HILIC columns equilibrate faster with more aqueous |
| Have the same ionic strength in both mobile phases | • Ionic strength gradients have more variability than constant ionic strength  
• Near 90-100% ACN, many buffers crash out, causing serious clogs |
| Increasing buffer concentration can improve peak shape and sample loadability | • High buffer concentrations can cause ion suppression when using MS detection |
| Follow good measurement practices when mixing buffers | • Retention can vary from bottle-to-bottle if eluent is not mixed accurately and consistently |
| Prepare samples in as much acetonitrile as possible and keep injection volumes small | • Avoid peak shape and retention issues from strong solvent effects |
| Use inert solution, if needed | • Reduce unwanted interactions of analytes with metal in the flow path |
HILIC Column Equilibration is Faster with Higher Amounts of Aqueous

B vitamins on Agilent InfinityLab Poroshell 120 HILIC-OH5 (2.1 x 100 mm, 2.7 μm)

4% aqueous, isocratic
Equilibrated in 30 minutes (75 column volumes)

20% aqueous, isocratic
Equilibrated in <10 minutes (<25 column volumes)

Gradient methods also equilibrate quickly due to higher amounts of aqueous mobile phase being used.

Column stored in 100% CH₃CN before analysis; A: 100 mM ammonium formate pH 3.0, B: CH₃CN, 96% B isocratic, 0.5 mL/min, 1 μL injection of B2+B6, 25 °C, 260 nm, 80 Hz

Column stored in 100% CH₃CN before analysis; A: 100 mM ammonium formate pH 3.0, B: CH₃CN, 80% B isocratic, 0.5 mL/min, 1 μL injection of B9+B12, 25 °C, 260 nm, 80 Hz
Higher Salt Concentrations Can Improve Peak Shapes and Resolution

Inorganic Ions on Agilent InfinityLab Poroshell 120 HILIC-Z

10 mM

I⁻, Br⁻, Cl⁻, Na⁺, K⁺, Mg²⁺, Ca²⁺, H₂PO₄⁻

50 mM

100 mM

Higher salt concentrations may reduce LC-MS sensitivity, choose the most appropriate mobile phase for your analysis.

Agilent InfinityLab Poroshell 120 HILIC-Z 2.1 x 100 mm, 2.7 μm; A: 10, 50, or 100 mM pH 3 ammonium formate, B: Acetonitrile, 80-20% B in 5 min, 3 min re-equilibration, 0.4 mL/min, 30 C, 2 μL injection of individual standards (0.3 to 0.5 mg/mL), ELSD 40 °C/3.5 psi/30Hz
HILIC Analyses Perform Best with Weak Injection Solvents

B vitamins on HILIC with isocratic elution

1 µL injection in H₂O
1 µL injection in H₂O/CH₃CN (3:1)
1 µL injection in H₂O/CH₃CN (1:1)
1 µL injection in H₂O/CH₃CN (1:3)
1 µL injection in CH₃CN

Agilent ZORBAX RRHD HILIC Plus 2.1 x 50 mm, 1.8 µm; Mobile phase: acetonitrile/100 mM ammonium formate pH 3.2 in water (9:1), isocratic elution, 0.4 mL/min, 1 µL injection of 5.7 µg/mL each of 4-aminobenzoic acid, nicotinamide, riboflavin, nicotinic acid; 25 °C, MS source: ESI+, 200 °C, 10 L/min, 30 psi, 4000 V; SIM: 138, 123, 377, 124
HILIC Sensitivity Can Be Improved with a PEEK-Lined Column

- Metal-free flow path minimizes unwanted interactions
- Stainless steel provides strength for UHPLC use

For best results, use the full InfinityLab bio-inert LC Solution:
- InfinityLab bio-inert LC System
- Bio-inert quick connect heat exchanger, p/n: G7116-60009
- All Agilent PEEK/SST Bio-inert capillaries with Quick Turn fitting (5067-5966) or UHP-FF fitting Bio-inert (5067-5695)
Tips and Tricks for Your HILIC Separations

InfinityLab deactivator additive pairs well with PEEK-lined HILIC-Z

<table>
<thead>
<tr>
<th>Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reduce Metal-Analyte Interaction</strong></td>
</tr>
<tr>
<td>• Chelate-free metals, covers exposed active sites in sample flow path, reducing unwanted metal-analyte interactions and allowing lower detection limits using LC/MS</td>
</tr>
<tr>
<td><strong>Amenable to LC/MS use</strong></td>
</tr>
<tr>
<td>• Optimized for use at a 5 µM (1:1000 dilution) with minimal ion suppression effects</td>
</tr>
<tr>
<td>• Does not persist in the LC/MS system after use (unlike traditional ion pairing reagents)</td>
</tr>
<tr>
<td><strong>Operational time and cost savings</strong></td>
</tr>
<tr>
<td>• Saves time needed to passivate your system</td>
</tr>
<tr>
<td>• Can avoid derivatization</td>
</tr>
<tr>
<td>• Can avoid potential system contamination from ion pairing agents</td>
</tr>
<tr>
<td>• Limits of detection can be lowered for challenging compounds such as phosphorylated metabolites, phosphate pesticides, and organic acids</td>
</tr>
</tbody>
</table>

InfinityLab deactivator additive
50 mL: 5190-4506

Recommended read

More information can be found in the InfinityLab Deactivator Additive user guide 5991-9516EN.
LC passivation procedure to reduce unwanted metal interactions

- LC disconnected from MS and going directly to waste
- IPA at 5 mL/min for 5 min
- Water at 5 mL/min for 5 min
  - Flow at 0.5 mL/min for 1 hour
- 0.5% phosphoric acid in 90% acetonitrile/10% water at 5 mL/min for 5 min
  - Flow at 0.1 mL/min overnight (at a minimum)
- Water at 5 mL/min for 5 min
  - Flow at 0.5 mL/min for 1 hour
- Mobile phase at 5 mL/min for 5 min
  - Flow at 0.25 mL/min for 1 hour
- Reconnect LC to MS and proceed with analysis
  - Flow at 0.25 mL/min for 20 to 30 min
Tips and Tricks for Your HILIC Separations

Stepwise improvements for metal sensitive analytes

**Thiamine diphosphate**

- **Before system passivation**
  - $T_f = 3.5$
  - Plates = 1,878

- **After LC passivation with 0.5% phosphoric acid in 9:1 acetonitrile/water**
  - $T_f = 3.0$
  - Plates = 18,015

- **Added InfinityLab deactivator additive to mobile phase**
  - $T_f = 1.4$
  - Plates = 52,100

- **Installed PEEK-lined HILIC-Z column**
  - $T_f = 1.4$
  - Plates = 60,095

**Acquisition time (min)**

6.0 6.2 6.4 6.6 6.8 7.0 7.2 7.4
HILIC Column Care

Cleaning a HILIC column:
• Use a strong HILIC solvent to clean HILIC columns
• Flush HILIC columns with 100% water
• If that is insufficient, add in 100 to 500 mM salt
  – You can use a strong salt like NaCl or, if you prefer to avoid that, you can use buffer salts like ammonium acetate
• Increasing the temperature to 35 to 55 °C can also help with the cleaning efficiency
• Flush with about 30 column volumes per step
• Be sure that once you have finished flushing with high concentration salt, you flush with pure water before reintroducing acetonitrile into the mobile phase

Storing a HILIC Column:
• Flush with acetonitrile/water (20/80) for 30 column volumes
• Flush with acetonitrile/water (80/20) for 30 column volumes
• Store at room temperature
Summary

When to consider a HILIC column:

- Are your analytes unretained with RPLC?
- Are your analytes at least somewhat soluble in acetonitrile?
- Are you using MS detection?
- Do your analytes interact with metals in the LC system?

Keep sample solvents in mind for HILIC analyses; prepare the sample in as much acetonitrile as possible and keep injection volumes as small as possible

- Most common support issue with HILIC methods
Additional Information

Learn more about Agilent HILIC Column portfolio
### Application notes on Poroshell 120 HILIC columns

#### Agriculture and Food Testing
- Analysis of Amino Acids in Animal Feed Matrices Using the Ultivo Triple Quadrupole LC/MS System – 5994-0586EN
- Analysis of Sugars Using an Agilent InfinityLab Poroshell 120 HILIC-Z Column – 5991-8984EN
- Analysis of Organic Acids on an Agilent InfinityLab Poroshell 120 HILIC-Z Column – 5991-8985EN
- LC/MS Analysis of Free Amino Acids on Agilent InfinityLab Poroshell 120 HILIC 1.9 μm Columns – 5991-7541EN

#### Biopharma
- Integrated Transcriptomics and Metabolomics Study of Retinoblastoma Using Agilent Microarrays and LC/MS/GC/MS Platforms – 5991-6215EN
- Enhanced Metabolite Profiling from Bark of Alangium Salviifolium Using LC/MS and GC/Q-TOF Techniques – 5991-4663EN
- Analysis of Water-Soluble Vitamins and their Metabolites – 5994-1553EN
- Methods for the Analysis of Underivatized Amino Acids by LC/MS – 5991-8582EN
- HPLC-DAD Analysis of Nucleotides Using a Fully Inert Flowpath – Agilent 1260 Infinity II Bio-inert LC System and a PEEK-Lined Agilent InfinityLab Poroshell 120 HILIC-Z Column – 5994-0680EN
- 13C Glucose Qualitative Flux Analysis in HEPG2 Cells Using an Agilent 6546 LC/Q-TOF and VistaFlux – 5994-0713EN
- Analysis of Choline Metabolites by Hydrophilic Interaction Chromatography (HILIC) with LC/MS/MS – 5991-9491EN
- Monitoring of Mammalian Cell Culture Media with HILIC LC/MS – 5994-0024EN

### Application notes on Poroshell 120 HILIC columns

#### Application Note Title

| Small Molecule Pharma | • Impurity Analysis of Aminoglycoside Antibiotic Using the Agilent InfinityLab Poroshell 120 HILIC-S Column with ELSD Detection – 5991-8824EN  
• Trace Level Quantification of Potential Mutagenic Impurities in Pharmaceuticals Using an Agilent Ultivo LC/TQ with Mixed Mode Detection – 5994-1238EN  
• How to Catch a Potential Mutagenic Impurity Using Agilent LC/MSD XT and Agilent InfinityLab Poroshell 120 HILIC-Z Column for Sensitive and Reliable Detection of Dalfampridine Impurities – 5994-0864EN  
• Analysis of Polar Compounds in Plant Material – 5991-8617EN  
• Analysis of Water-Soluble Vitamins on an Agilent InfinityLab Poroshell 120 HILIC-OH5 Column – 5991-8780EN  
• Analysis of Aminoglycosides Using the Agilent InfinityLab Poroshell 120 HILIC-Z Column – 5991-8824EN |  |
| Environmental | • Paraquat, Diquat, and Mepiquat Analysis in Environmental Water – 5994-1307EN  
• Modified QuEChERS for HILIC LC/MS/MS Analysis of Nicotine and its Metabolites in Fish – 5991-2408EN  
• Analysis of Metals, Halides, and Inorganic Ions Using Hydrophilic Interaction Chromatography – 5991-8602EN |  |
| General | • Retaining and Separating Polar Molecules – A Detailed Investigation of When to Use HILIC versus a Reversed-Phase LC Column – 5994-1137EN  
• Hydrophilic Interaction Chromatography (HILIC) Using Agilent Poroshell 120 HILIC – 5991-1242EN  
• Hydrophilic Interaction Chromatography Method Development and Troubleshooting – 5991-9271EN  
• Analysis of Highly Polar Compounds by SFC/Q-TOF MS with Identification using Database and Library Searches – Enhanced Fluidity Liquid Chromatography (EFLC) using High Modifier Concentration at Elevated System Pressure – 5994-1096EN |  |

Resources for Support

- LC troubleshooting poster (5994-0709EN)
- Tech support www.agilent.com/chem/techsupport
- Resource page www.agilent.com/chem/agilentresources
  - Quick reference guides
  - Catalogs, column user guides
  - Online selection tools, how-to videos
  - Application workflows (such as cannabis, PFAS, and more)
- InfinityLab LC Supplies catalog (5991-8031EN)
- LC handbook (5990-7595EN)
- Best practices for using an Agilent LC system (01200-90090)
- Your local FSE and specialists
- Agilent University www.agilent.com/crosslab/university
- YouTube – Agilent Channel (maintenance videos)
- Agilent service contracts
Contact Agilent Chemistries and Supplies Technical Support

1-800-227-9770 option 3, option 3:
Option 1 for GC and GC/MS columns and supplies
Option 2 for LC and LC/MS columns and supplies
Option 3 for sample preparation, filtration, and QuEChERS
Option 4 for spectroscopy supplies
Option 5 for chemical standards
Option 6 for former Prozyme products

Available in the U.S. and Canada, 8–5 all time zones

gc-column-support@agilent.com
lc-column-support@agilent.com
spp-support@agilent.com
spectro-supplies-support@agilent.com
chem-standards-support@agilent.com
advancebio.glycan@agilent.com

Web chat: Product pages of agilent.com
Thank you
Appendix

Agilent applications
Analysis of Amino Acids (and Isobars) in Plant Tissue with LC-MS/MS

1. Aspartic acid
2. Phenylalanine
3. Leucine
4. Isoleucine
5. Methionine
6. Valine
7. Proline
8. Tyrosine
9. Cysteine
10. Alanine
11. Homoserine
12. Threonine
13. Glycine
14. Glutamine
15. Asparagine
16. Glutamic acid
17. Citrulline
18. Histidine
19. Lysine
20. Ornithine

1 µL of 500 ng/mL standard
Agilent publication: 5991-8922EN

Agilent publication: 5991-8922EN
High Throughput LC/MS Analysis of Amino Acids with an Agilent InfinityLab Poroshell 120 HILIC-Z Column

InfinityLab Poroshell 120 HILIC-Z
2.1 x 50 mm
A: 10% 200 mM ammonium formate pH 3.5, 90% H₂O
B: 10% 200 mM ammonium formate pH 3.5, 90% ACN
0.5 mL/min

T (min) | %B
---|---
0 | 97
2 | 90
5 | 70
5.5 | 70
5.6 | 97
7.5 | 97
Water Soluble Vitamins on Agilent InfinityLab Poroshell 120 HILIC-Z

System: Agilent 1260 Infinity Binary HPLC w/ DAD
Column: InfinityLab Poroshell 120 HILIC-Z, 2.1 x 100 mm (p/n: 685775-924)
Flow Rate: 0.5 mL/min
Column Temperature: 40 °C
Injection Volume: 1 μL
Mobile Phase A: 100 mM ammonium acetate + 0.5% acetic acid in water
Mobile Phase B: Acetonitrile
Wavelength: 260 nm
Data Rate: 80 Hz
Gradient: 87% B for 1 min, 87-50% B in 4 min, 3 min re-equilibration
Sample: Water Soluble Vitamin Standards (individual): 0.1-0.4 mg/mL

InfinityLab Poroshell 120 HILIC-Z
Agilent publication: 5991-8780EN
Organic Acids on Agilent InfinityLab Poroshell 120 HILIC-Z

Agilent 1260 Infinity Binary HPLC with DAD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>InfinityLab Poroshell 120 HILIC-Z, 2.1 x 100 mm, 2.7 μm (p/n: 685775-924)</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.5 mL/min</td>
</tr>
<tr>
<td>Column Temperature</td>
<td>30 °C</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>1 μL</td>
</tr>
<tr>
<td>Mobile Phase</td>
<td>30% 30 mM sodium phosphate + 0.075% phosphoric acid, pH ~6.7, 70% ACN*</td>
</tr>
<tr>
<td>Wavelength</td>
<td>214 nm</td>
</tr>
</tbody>
</table>

- Acetic acid
- Butyric acid
- Lactic acid
- Citric acid
- Ascorbic acid
- Sorbic acid
- Succinic acid
- Fumaric acid
- Oxalic acid
- t-Aconitic acid

*Sodium phosphate is not soluble in high % ACN.

- Do not increase salt concentration in mobile phase A.
- Do not increase %B
- If using ELSD or MS, use similar pH/concentration ammonium acetate instead

Agilent publication: 5991-8985EN
Separation of 11 Sugars on Agilent InfinityLab Poroshell 120 HILIC-Z

Mobile phase A: 0.3% ammonium hydroxide in H₂O
Mobile phase B: 0.3% ammonium hydroxide in CH₃CN,
85-60% B in 6 min, 0.4 mL/min, 35 °C, ELSD: 60 °C, 3.5 psi
2.1 x 100 mm, 2.7 µm Agilent InfinityLab Poroshell 120 HILIC-Z

1. Xylose
2. Arabinose
3. Fructose
4. Mannose
5. Glucose
6. Galactose
7. Sucrose
8. Maltose
9. Lactose
10. Maltotriose
11. Raffinose

Agilent publication: 5991-8984EN
InfinityLab Poroshell 120 HILIC-Z Analysis of Paraquat/Diquat

Column: InfinityLab Poroshell 120 HILIC-Z, 2.1 x 100 mm

Mobile Phase A: 20 mM ammonium formate in water, pH=3
Mobile Phase B: 20 mM ammonium formate in 90% acetonitrile in water, pH=3
Flow Rate: 0.80 mL/min
Column Temperature: 30 °C
Injection Volume: 0.25 µL
Total Runtime: 16 min

Parameter Setting:
- Mass Spectrometer: 6470 LC/QQQ in dMRM mode
- Ionization Mode: Jet Stream positive
- Gas Temp: 300 °C
- Gas Flow: 7.0 L/min
- Nebulizer: 45 psi
- Sheath Gas Temp: 400 °C
- Sheath Gas Flow: 11 L/min
- Capillary Voltage: 3500 V
- Nozzle voltage: 0 V

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Percentage B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>10</td>
<td>70</td>
</tr>
<tr>
<td>11</td>
<td>100</td>
</tr>
</tbody>
</table>

Analyte Concentration (mM), Precursor Ion (m/z), Product Ion (m/z), Fragmentor (V), Dwell Time (ms):
- Paraquat: 0.25, 185.1, 170.1, 100, 10
- Diquat: 0.25, 183.1, 157.1, 100, 10

Agilent Pub # 5991-8830EN
Analysis of Polar Compounds in Plant Materials: Quantitation of Stachydrine in Chinese Motherwort (Leonurus japonicas) by InfinityLab Poroshell 120 HILIC-Z

### Agilent 1260 Infinity II HPLC
- **Column**: InfinityLab Poroshell 120 HILIC-Z, 2.1 x 100 mm (p/n 685775-924)
- **Mobile Phase A**: 10 mmol ammonium acetate
- **Mobile Phase B**: Acetonitrile
- **Flow Rate**: 0.30 mL/min
- **Gradient**: 95-60% B in 10 min
- **Column Temperature**: 30 °C
- **Injection Volume**: 2 µL

### Agilent 1290 Infinity II ELSD
- **Nebulizer Temperature**: 40 °C
- **Evaporator Temperature**: 40 °C
- **Gas flow rate**: 1.6 SLM
- **Data Rate**: 40 Hz

Agilent publication: 5991-8617EN
Analysis of Metals, Halides, and Inorganic Ions on Agilent InfinityLab Poroshell HILIC-Z

Agilent 1260 Infinity Binary HPLC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>InfinityLab Poroshell 120 HILIC-Z, 2.1 x 100 mm, 2.7 μm (p/n 685775-924)</td>
</tr>
<tr>
<td>Flow Rate</td>
<td>0.40 mL/min</td>
</tr>
<tr>
<td>Column Temperature</td>
<td>30 °C</td>
</tr>
<tr>
<td>Injection Volume</td>
<td>1 μL</td>
</tr>
<tr>
<td>Mobile Phase A</td>
<td>100 mM ammonium formate in water at pH=3</td>
</tr>
<tr>
<td>Mobile Phase B</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Gradient</td>
<td>91% B for 1 min, 91-80% B in 5 min, 80-20% B in 5 min, 3 min re-equilibration</td>
</tr>
</tbody>
</table>

Agilent G4218A ELSD

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature</td>
<td>40 °C</td>
</tr>
<tr>
<td>Pressure</td>
<td>3.5 psi</td>
</tr>
<tr>
<td>Data Rate</td>
<td>30 Hz</td>
</tr>
</tbody>
</table>

Samples:
- **Calcium chloride**
- **Magnesium chloride**
- **Potassium bromate**
- **Potassium iodide**
- **Potassium phosphate**
- **Sodium bromide**
- **Sodium chlorate**